Mitotic Assay Kit (Colorimetric and Chemiluminescent)

(version A)

Catalog No. 18021 (Colorimetric format)

Catalog No. 18022 (Chemiluminescent format)

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Introduction

Active Motif's Mitotic Assay Kit enables you to determine the percentage of cells that are undergoing mitosis within a specified population. The Mitotic Assay Kit includes a phospho-histone H3 (Ser 28) monoclonal antibody (clone HTA28) and a HRP-conjugated secondary for detection. Crystal Violet is included as a stain to normalize for cell number. Paclitaxel is included to use as a positive control treatment to generate a high-mitotic reference population.

Cell division is a complex and tightly regulated process that is marked by the process of mitosis. During the progression of interphase in stage G2 to M phase, chromatin is packed into condensed chromosomes for nuclear division to continue. During this event, histones may be phosphorylated in a specific and temporally regulated manner. Recently, studies have shown that histone H3 is specifically phosphorylated at serines 10 and 28 during mitotic chromatin condensation[1,2]. Therefore, the phosphorylated histone H3 serine 28 residue is a useful marker for mitosis and this specific phosphorylation site can be used to determine the mitotic index of a population of cells.

The mitotic index, or proportion of cells undergoing mitosis within a specified cell population, is easily determined by calculating the percentage of cells positive for the histone H3 (Ser 28) signal relative to the untreated control cells. The total number of cells can be determined by the crystal violet stain. The phospho-histone H3 (Ser 28) is detected by the HRP-conjugated secondary antibody and subsequent colorimetric or chemiluminescent development. The colorimetric or chemiluminescent signal reveals cells undergoing mitosis (Figures 1 and 2).

The drug paclitaxel prevents mitotic spindle assembly and results in cells that are arrested during mitosis in late G2/M phase to provide a reading for high-mitotic index within a treatment population. This treatment provides a reference to compare the effect of test compounds on mitosis.

product	format	catalog no.
Mitotic Assay Kit (Color)	2 x 96 reactions	18021
Mitotic Assay Kit (Chemi)	2 x 96 reactions	18022

Kit Performance and Benefits

The Mitotic Assay Kit is for research use only. Not for use in diagnostic procedures.

Antibody Specificity

The anti-phospho-Histone H3 (Ser 28) clone HTA28 is raised in rat and generated against a synthetic peptide corresponding to residues 23-35 with a phosphorylated serine 28 residue of human histone H3.

The antibody cross reacts with human, mouse, hamster and bovine samples and is expected to cross react with other mammalian species based on sequence homology.

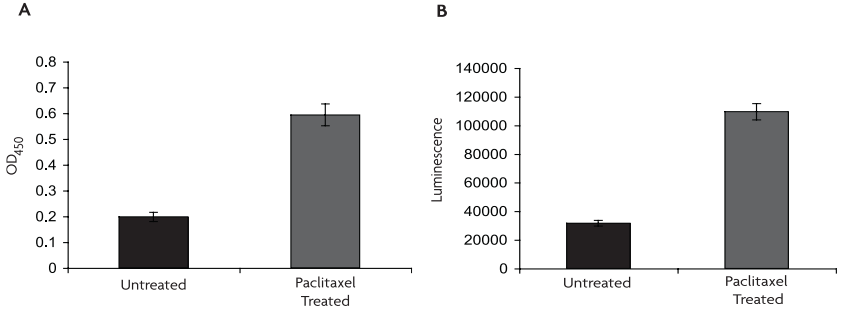

Figure 1: Colorimetric (Figure 1A) and Chemiluminescent (Figure 1B) results.

HeLa cells were treated with 1 µM paclitaxel for 6 hours. Results are the average from 8 wells of a 96-well plate.

Kit Components and Storage

Store components at the temperatures indicated below. Paclitaxel is provided resuspended in DMSO and will be frozen when stored at 4°C. Aliquot Paclitaxel to avoid subjecting to multiple freeze-thaws. The antibodies may also be aliquoted and stored at -20°C for long-term storage.

Reagents Common to Both Kits	Quantity	Storage / Stability
anti-phospho-Histone H3 (Ser 28)	15 µl	4°C for 6 months
Goat anti-Rat IgG-HRP	2 x 12 µl	4°C for 6 months
Paclitaxel (1 mM)	15 µl	4°C for 6 months
1X Antibody Blocking Buffer	22 ml	-20°C for 6 months
1X Antibody Dilution Buffer	30 ml	-20°C
10X PBS	120 ml	room temp to -20°C
10% Triton X-100	7 ml	room temperature
Crystal Violet Solution	22 ml	4°C
1% SDS Solution	22 ml	room temperature
96-well tissue culture plate*	2	
Plate sealing tape	2	

Colorimetric Kit Developing Reagent	Quantity	Storage / Stability
Developing Solution	22 ml	4°C
Stop Solution	22 ml	4°C

Chemiluminescent Kit Developing Reagent	Quantity	Storage / Stability
Chemiluminescent reagent	4 ml	4°C
Reaction buffer	8 ml	4°C

*Suitable tissue culture plates for the colorimetric assay are Greiner part no. 655180 and Corning Costar part no. 3596.

*Suitable tissue culture plates for the chemiluminescent assay are Greiner part no. 655098 and Corning Costar part no. 3596.

Additional materials required

- Colorimetric assay requires microplate spectrophotometer capable of reading at 595 nm and at 450 nm (655 as optional reference wavelength)
- Chemiluminescent assay requires a luminometer
- multi-channel pipette and pipettor reservoirs
- rocking platform
- parafilm

- fresh 10% hydrogen peroxide (3 ml required)
- 10 mg/ml poly-L-Lysine (if using non-adherent cells)
- 10% sodium azide (250 µl required)
- 37% formaldehyde (2.5 ml for adherent cells, 5 ml for non-adherent cells)

WARNING: Sodium Azide and Formaldehyde are highly toxic chemicals. Appropriate safety precautions (gloves and eye protection) should be used. In addition, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood.

Buffer & Reagent Preparation

Positive control treatment

Paclitaxel

Dilute the 1 mM Paclitaxel stock solution 1:1000 in complete cell medium to make a 1 µM final concentration. Remove existing medium and add 200 µl of the paclitaxel solution to each well.

Buffer preparation

We provide an excess of buffer components in order to perform 2 x 96-well Mitotic Assays with the phospho-histone H3 (Ser 28) antibody. Required reagents that are not supplied are listed on the previous page.

Preparation of 1X PBS

1X PBS is the basis of several buffers used in the FACE protocol. 1X PBS is also used in several of the wash steps in the protocol. It is prepared by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH_2O and mixing thoroughly.

Preparation of Fixing Buffer (4% or 8% Formaldehyde in PBS)

Fixing Buffer is used to fix cells after cell culturing. It is prepared by adding formaldehyde to 1X PBS and mixing well. 4% formaldehyde is used with adherent cells, 8% formaldehyde is used with non-adherent cells. For example, for one plate, dilute 1.3 ml paraformaldehyde to 10.7 ml PBS for a final 4% solution.

Preparation of Wash Buffer (0.1% Triton X-100 in PBS)

Wash Buffer is used throughout the FACE protocol and is prepared by adding the provided 10% Triton X-100 solution to 1X PBS and mixing thoroughly. For 192 wells, dilute 6.26 ml 10% Triton X-100 to 620 ml 1X PBS.

Quenching Buffer (Wash Buffer containing 1% H_2O_2 and 0.1% Azide)

Quenching Buffer is used to inactivate the cells' endogenous peroxidase activity. It is prepared by adding fresh Sodium Azide and fresh hydrogen peroxide to the Wash Buffer.

Blocking Buffer

This is supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

Antibody Dilution Buffer

This is supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

Diluted phospho-Histone H3 (Ser 28) antibody

The antibody will be diluted 1:2000 in Antibody Dilution Buffer. For 2 plates, dilute 5 µl of the antibody in 10 ml of the Antibody Dilution Buffer.

Diluted HRP-conjugated secondary antibody

HRP-conjugated anti-rabbit IgG is used as the secondary antibody to detect bound primary antibodies. The supplied antibody will be diluted 1:1000 in Antibody Dilution Buffer.

1% SDS Solution

1% SDS Solution is used in the Crystal Violet counting procedure to solubilize cells and release the dye for subsequent quantification at 595 nm. This buffer is supplied ready-to-use.

Crystal Violet Solution

This is supplied ready-to-use. Crystal Violet is used to determine the relative number of cells in each well. This stain binds to cell nuclei and gives an OD_{595} reading that is proportional to cell number.

Specific reagents for Colorimetric Kit

Developing Solution

The Developing Solution must be warmed to room temperature before use. This solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the solution indicates that it has been contaminated and must be discarded. Prior to use, transfer the amount of Developing Solution required for the assay into a secondary container, avoid direct exposure to intense light and leave at room temperature for at least 1 hour. After use, discard any remaining solution that was transferred into the secondary container.

Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard any remaining Stop Solution that was transferred into the secondary container.

WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* labcoat, gloves and eye protection.

Specific reagents for Chemiluminescent Kit

Preparation of Chemiluminescent Working Solution

The Chemiluminescent Reagent and Reaction Buffer should be warmed to room temperature before use. These components are light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. Prior to use, place the Chemiluminescent Reagent and Reaction Buffer at room temperature for at least 1 hour. In a separate container, mix 1 volume of Chemiluminescent Reagent with 2 volumes of Reaction Buffer to prepare the Chemiluminescent Working Solution. The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Step 1: Culture, fix and block the cells

Note: This protocol is the same for both chemiluminescent and colorimetric assay kits until Step 3, which is the detection portion of the assay.

1. Seed the cells in the 96 well plate so that they will be approximately 80% confluent at the time of fixing, after they have been treated as desired. The growth area in each well of the plate is 0.32 cm^2. The provided plates are sterile and treated for tissue culture.
2. Grow and treat cells as desired. For the positive control, a 6 hour treatment with paclitaxel at 37°C the day after the cells were seeded is recommended.

   Note: Depending on experimental design, some wells should be incubated with the positive control, paclitaxel, some wells should be left untreated and the remaining wells can be treated with test compounds. Cell lines that divide slowly may require a longer treatment with paclitaxel to observe a clear increase in the mitotic index.

3. Fix cells by replacing the growth medium with 100 µl of 4% formaldehyde in PBS. To minimize the escape of formaldehyde vapors, place a 10 cm x 17 cm piece of parafilm over the plate with the lid. The covered plate can also be placed in a zip-lock bag. Incubate for 20 minutes at room temperature.

   WARNING: Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical.

4. Remove formaldehyde solution and wash the cells 3 times with 200 µl Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
5. Remove Wash Buffer, add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.
6. Remove Quenching Buffer and wash cells 2 times for 5 minutes with 200 µl Wash Buffer.
7. Remove Wash Buffer, add 100 µl Antibody Blocking Buffer and incubate for 1 hour at room temperature.

Step 2: Binding of primary and secondary antibodies

1. Remove Antibody Blocking Buffer and wash cells 2 times with 200 µl Wash Buffer.
2. Remove Wash Buffer, add 50 µl of diluted primary antibody (or Antibody Dilution Buffer for negative control wells) and seal the plate with sealing tape. Place a 10 cm x 17 cm piece of parafilm over the plate, cover with lid and incubate overnight at 4°C or for 1 hour at room temperature. Be sure that the plate is level and that each well is tightly sealed with the sealing tape to prevent evaporation.

3. Remove primary antibody, wash cells 3 times for 5 minutes each with 200 µl Wash Buffer.
4. Remove the Wash Buffer, add 100 µl diluted secondary antibody, cover plate with tissue culture plate lid or sealing tape, and incubate 1 hour at room temperature.

   Note: During this second incubation, warm detection reagents to room temperature.

   For the colorimetric kit, transfer the amount of Developing Solution required for the assay into a secondary container and leave at room temperature for at least an hour (avoid light).

   For the chemiluminescent kit, place the chemiluminescent working solution and reaction buffer at room temperature.

5. Remove secondary antibody, wash cells 3 times for 5 minutes with 200 µl Wash Buffer and then 2 times for 5 minutes with 200 µl 1X PBS. Proceed to either the colorimetric (step 3a) or chemiluminescent (step 3b) detection steps depending on which kit you are using.

Step 3a: Colorimetric reaction (not for chemiluminescent kits)

1. Remove secondary antibody, wash cells 3 times for 5 minutes with 200 µl Wash Buffer and then 2 times for 5 minutes with 200 µl 1x PBS.
2. Remove the PBS from the wells and then add 100 µl Developing Solution to each well.
3. Incubate for 2-20 minutes at room temperature protected from direct light. Monitor the blue color development until the darkest-staining wells are medium- to dark blue. Do not over-develop.
4. Add 100 µl Stop Solution. This acidic solution turns the blue color to yellow. Take care with pipetting to ensure that each well is developed for the same amount of time.

   WARNING: The stop solution is corrosive. Wear personal protective equipment when handling (i.e. lab coat, gloves and eye protection).

5. Read absorbance on a spectrophotometer within 5 minutes at 450 nm with an optional reference wavelength of 655 nm.

Step 3b: Chemiluminescent detection (not for colorimetric kits)

1. Remove secondary antibody, wash cells 3 times for 5 minutes with 200 µl Wash Buffer and then 2 times for 5 minutes with 200 µl 1x PBS.
2. Remove the PBS from the plate wells and add 50 µl room temperature Chemiluminescent Working solution to each well.
3. Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 10 minutes to minimize changes in signal intensity.

Optional – Crystal Violet cell staining

Crystal Violet is an intense stain that binds to the cell nuclei and gives an OD595 reading that is proportional to cell number. To normalize the readings for the mitotic index assay follow the steps below.

1. After reading the plate at 450 nm, wash wells twice with 200 µl Wash Buffer and 2 times with 200 µl 1x PBS. Tap plates onto paper towels to remove excess liquid from wells and air-dry at room temperature for 5 minutes.
2. Add 100 µl Crystal Violet solution to each well and incubate 30 minutes at room temperature.

   WARNING: Crystal Violet is an intense purple stain. Avoid contact with skin and clothing.
3. Wash wells 3 times with 200 µl 1x PBS for 5 minutes each.
4. Add 100 µl of 1% SDS Solution to each well and incubate on a shaker for 1 hour at room temperature.
5. Read absorbance on a spectrophotometer at 595 nm. If the signals obtained are greater than the range of the spectrophotometer, the signal can be reduced by removing some (e.g. 50 µl) of the liquid from each well and replacing it with an equivalent volume of dH2O.
6. The measured OD450 readings are corrected for cell number by dividing the OD450 reading for a given well by the OD595 reading for that well.

References

1. Goto, H. *et al.* (1999) *J. Biol. Chem.* 274(36): 25543-25549.
2. Hirata, A. *et al.* (2004) *J. Histochemisty & Cytochemistry* 52(11): 1503-1509.

Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in wells incubated with antibody	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers
	Plate reader or CCD camera settings not optimal	Verify the wavelength (measurement mode) and filter settings in the plate reader
	Developing Solution was cold	Bring Developing Solution to room temperature
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
	Insufficient number of cells were plated	Plate cells so that they are 80% confluent at time of fixing
	Cells did not adhere correctly to plate	Follow protocol for use of non-adherent cells
	Excessive washing	Wash steps should be 5 minutes each
	Incubation of secondary antibody was too long	Incubate secondary antibody for 1 hour
Little or no signal is observed from detecting anti-phospho Histone H3 (Ser 28)	Cells are dividing slowly. This could be due to recent thaw, ill-health or cell type.	Do not use cells that have been recently thawed.
		Make sure cells have adequate nutrients and CO_2 and are maintained at 37°C.
		Change to a cell line that divides more rapidly.
No increase in mitotic index is observed after paclitaxel treatment.	Cells are dividing too slowly to be arrested in mitosis.	Increase the length of time that cells are incubated with paclitaxel. If treating overnight, decrease paclitaxel concentration to 250 to 500 nM to avoid cyto-toxicity.
		Change cell model.

High background in all wells	Developing time too long (Colorimetric Assay)	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue
	Measurement time too long (Chemiluminescent Assay)	Reduce integration time or exposure time on luminometer or CCD camera
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start with 1 in 2000 for the primary antibody. If the background is still high, then try the secondary antibody at 1 in 2000, while keeping the primary at 1 in 2000. Different cell lines react differently with the antibodies.
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Inadequate quenching or blocking	Ensure that quenching and blocking steps were performed according to the protocol
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
Antibody solution evaporates from well during overnight incubation with primary antibody	Sealing tape was incorrectly applied	Ensure that each well is sealed when sealing tape is applied and ensure that the parafilm sheet covers the plate completely before the lid is placed on the plate. The plate can also be placed in a zip-lock or heat-sealed bag
Insufficient sensitivity	Antibody concentration incorrect	If the cells studied have very low levels of the protein of interest, the sensitivity of detection may be improved by increasing the concentration of primary antibody used and by minimizing the incubation volume. It is possible to perform the overnight incubation in as little as 25 µl, however, this will make multichannel pipetting difficult and requires the plate be carefully sealed and incubated on a level surface. Alternatively, if the cells have easily detectable levels of the phosphorylated protein and the detection of small changes in phosphorylation is desired, sensitivity of the assay may be improved by decreasing the concentration of the phospho antibody used

Section B. Related Products

Mitotic Index Assay	Format	Catalog No.
Mitotic Index Assay Kit (Fluorescent)	5 x 96 rxns	18020

Fluorescent Dyes	Format	Catalog No.
Chromeo™ 488 NHS-Ester	1 mg	15511
	5 mg	16511
Chromeo™ 494 NHS-Ester	1 mg	15111
	5 mg	16111
Chromeo™ 546 NHS-Ester	1 mg	15211
	5 mg	16211
Chromeo™ 642 NHS-Ester	1 mg	15311
	5 mg	16311

Fluorescent Secondary Antibodies	Format	Catalog No.
Chromeo™ 488 Goat anti-Mouse IgG	1 mg	15031
Chromeo™ 488 Goat anti-Rabbit IgG	1 mg	15041
Chromeo™ 494 Goat anti-Rabbit IgG	1 mg	15042
Chromeo™ 546 Goat anti-Mouse IgG	1 mg	15033
Chromeo™ 546 Goat anti-Rabbit IgG	1 mg	15043
Chromeo™ 642 Goat anti-Mouse IgG	1 mg	15034
Chromeo™ 642 Goat anti-Rabbit IgG	1 mg	15044

Fluorescent Cell Stain	Format	Catalog No
LavaCell™	200 µg	15004

Protein Labeling	Format	Catalog No.
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ Fluorescein Label	300 rxns	34101
LigandLink™ Hexachlorofluorescein Label	300 rxns	34104
LigandLink™ pLL-1-NFκB p65 Kit	1 kit	34004
LigandLink™ pLL-1-p53 Kit	1 kit	34005
LigandLink™ pLL-1-STAT1 Kit	1 kit	34006

Luciferase Assays	Format	Catalog No.
RapidReporter® Gaussia Luciferase Assay	100 rxns	33001
	1000 rxns	33002
RapidReporter® pRR-High vector	10 µg	33003
RapidReporter® pRR-High Assay	100 rxns	33004
RapidReporter® pRR-Low vector	10 µg	33005
RapidReporter® pRR-Low Assay	100 rxns	33006
RapidReporter® pRR-High-CRE vector	10 µg	33007
RapidReporter® pRR-High-CRE Assay	100 rxns	33008
RapidReporter® pRR-High-NFκB vector	10 µg	33009
RapidReporter® pRR-High-NFκB Assay	100 rxns	33010
RapidReporter® pRR-High-GR vector	10 µg	33011
RapidReporter® pRR-High-GR Assay	100 rxns	33012
RapidReporter® pRR-High-STAT3 vector	10 µg	33013
RapidReporter® pRR-High-STAT3 Assay	100 rxns	33014

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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